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Fatty Acid Acylated Proteins of the Halotolerant Alga *Dunaliella salina*[1]

Martha Stephenson, Phillip E. Ryals, and Guy A. Thompson, Jr.*
Department of Botany, The University of Texas, Austin, Texas 78713

ABSTRACT

The unicellular, wall-less alga *Dunaliella salina* has been shown to contain an array of proteins modified by the covalent attachment of fatty acids. Myristic acid (14:0) comprised approximately 80% by weight of the protein-linked acyl groups in samples derived from cells cultured in medium containing 1.7 molar NaCl and 93% in samples from cells grown in medium containing 3.0 molar NaCl. Palmitic and stearic acids accounted for most of the remaining protein-bound acyl chains. Approximately 0.2% of the incorporated radioactivity was estimated to be in linkage with protein. The bulk of acyl chains (about 99%) were resistant to cleavage by alkali, indicating a preponderance of amide bonding. The sodium dodecyl sulfate-polyacrylamide electrophoresis labeling pattern of proteins from [^{3}H]myristic-labeled cells was significantly different from that of proteins from cells exposed to [^{3}H]palmitate. The appearance of radioactivity in certain proteins was also influenced by the salinity of the culture medium. Thus growth in moderate (1.7 molar) salt favored the acylation of a 48-kilodalton polypeptide whereas in high (3.0 molar) salt, a 17-kilodalton polypeptide was more heavily labeled.

Proteins possessing covalently linked fatty acids are receiving increasing attention in the literature (12). These studies show myristate (14:0) and palmitate (16:0) to be the fatty acids most commonly bound to proteins, while stearate (18:0), oleate (18:1), and linoleate (18:2) have been reported in much smaller amounts (2, 8). Palmitate, generally found in ester linkage (7, 14), appears to be attached almost exclusively to membrane proteins while 14:0, commonly bound in amide linkage, is present in both membrane bound and soluble proteins. It is known that protein acylation can occur either co- or post-translationally (13, 19), but the physiological significance of the process remains unclear.

Studies of viral, mammalian and, recently, lower eukaryotic systems, such as *Tetrahymena* (17) have formed the basis of currently available information on acylated proteins. The first and, to our knowledge, the only characterization of acylated protein from a plant is found in the work of Mattoo and Edelman (9), who reported the palmitoylation of the chloroplast 32-kD herbicide-binding protein of *Spirodela oligorrhiza*. In this paper we report the presence of an array of acylated proteins in the halotolerant green alga *Dunaliella salina* grown in medium containing either 1.7 or 3.0 M sodium chloride. Data are also presented regarding the relative amounts of the acyl chain lengths detected and their linkage characteristics.

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MATERIALS AND METHODS

Culture Conditions, *in Vivo* Labeling of Acylated Proteins, and Protein Extraction/Delipidation

Axenic cultures of *Dunaliella salina* (UTEX No. 1644) were grown at 30°C, under continuous light in 500 mL Erlenmeyer flasks containing 250 mL of sterile synthetic medium as previously described (6). Sodium chloride was included to yield final salt concentrations of either 1.7 or 3.0 M. Cell density was determined using a Coulter counter model ZB. Cells in the 1.7 M NaCl-containing medium had a doubling time of approximately 20 h while those in the higher salt concentration grew only half as fast.

Cultures containing approximately 10^6 cells/mL were labeled by adding 0.5 mCi of either [9,10-^{3}H]myristic acid (22.4 Ci/mmol, DuPont/NEN, Boston, MA) or [9,10-^{3}H]palmitic acid (28.5 Ci/mmol, DuPont/NEN, Boston, MA) in a small volume of ethanol. Cells were allowed to incubate in the presence of label for 3 h, then washed twice by centrifugation (300*g* for 5 min) and resuspended in 250 mL of fresh, sterile medium. Incubation in the fresh medium was continued for an additional 3 h. Cells were again pelleted and 1 mL of electrophoresis sample buffer (17) (lacking β-mercaptoethanol) was added. Aliquots of this suspension were boiled in microfuge tubes for 5 min. Following the initial solubilization, samples were transferred to chilled Corex tubes, and 5 volumes of cold acetone were added. Subsequent delipidation of the precipitated protein was as described previously (17), utilizing seven extractions with chloroform:methanol (2:1, v/v) interspersed with two extractions with chloroform:methanol:concentrated HCl (6.7:3.3:0.1, v/v/v), with the release of free lipid radioactivity being monitored in each of the extracts. The delipidated protein samples were dried briefly under N_2, resolubilized in sample buffer (lacking β-mercaptoethanol), and assayed for protein by the bicinchoninic acid method (18). The presence of fatty acids covalently linked to proteins through a glycophospholipid anchor (5) was sought by incubating delipidated protein with a phosphatidylinositol-specific phospholipase C from *Bacillus thuringiensis* generously supplied by Dr. Martin G. Low, Columbia University, using a slight modification of the procedure of Ferguson *et al.* (3).

Analytical Procedures

Samples of delipidated protein were subjected to acid methanolysis in methanol:concentrated HCl, (8.3:1.7, v/v) at 100°C for 72 h to release all covalently bound fatty acids. The released fatty acid methyl esters were removed by 2 hexane extractions, and the solvent was evaporated under a stream of N_2. The residue was taken up in a small volume of methylene chloride for analysis by gas-liquid chromatography using the conditions and controls of Ryals and Thompson (16).

HPLC and radioactivity monitoring of individual fatty acids was performed on *p*-nitrophenacyl derivatives prepared from the released fatty acid methyl esters. Conditions for preparation of derivatives and HPLC analysis have been described (16, 17).

To estimate the relative amounts of ester and amide linkages, delipidated protein was treated with either 1 M hydroxylamine (pH 10) or 1 N KOH. We have shown the KOH procedure to be 90% effective in cleaving ester linked [^{14}C] 18:0 from authentic stearoyl-CoA (17). Radioactivity of the fatty acids released from ester linkage was measured by scintillation counting.

Fifty μg aliquots of delipidated protein were separated on either 12.5 or 20% acrylamide slab gels under denaturing conditions as described by Laemmli (4) at a constant 150 V. Gels were stained with either Coomassie brilliant blue or silver stain. Nondelipidated and delipidated whole cell proteins gave virtually identical staining patterns, except that the latter preparations contained some highly aggregated protein remaining at the top of the gel. Some gels were prepared for fluorography using either PPO-DMSO or Resolution (EM Corp., Chestnut Hill, MA) and were exposed to Kodak AR X-Omat x-ray film for up to 14 d at −70°C.

RESULTS AND DISCUSSION

Acylation of Proteins

Cells that had been exposed to tracer amounts of ^{3}H-fatty acid were delipidated by sequential extraction with organic solvents as described in "Materials and Methods." The last traces of noncovalently bound lipids were eventually removed through exhaustive extraction, as evidenced by the absence of radioactivity in the last three wash volumes. Approximately 0.2% of the total incorporated radioactivity was recovered in fully delipidated protein from cells grown in the normal 1.7 M NaCl and from those grown in 3.0 M NaCl medium.

SDS-PAGE and Fluorography

Figure 1 illustrates the whole cell polypeptide pattern on 12.5% acrylamide gels and fluorograms of gels run with delipidated protein from cells labeled with radioactive fatty acids. Fluorograms of proteins from cells labeled with [^{3}H] 14:0 indicated that radioactivity was associated with a broad range of polypeptides having approximate mol wt of 80, 72, 54, 48.5, 38, 23, 17.2, 15.8, 11, and 9 kD.

Relatively small but reproducible differences in labeling intensity were observed in cells labeled with [^{3}H]14:0 in 1.7 and 3.0 M NaCl. Thus, in 1.7 M NaCl, a 48 kD polypeptide

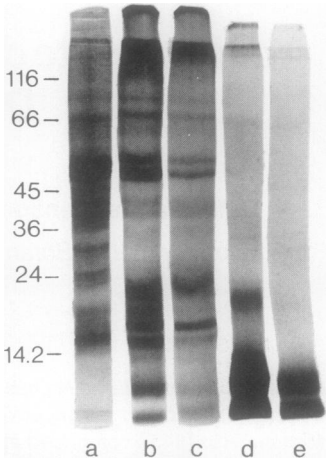

Figure 1. Electrophoretic gel patterns of *D. salina* whole cell proteins. The Coomassie blue staining pattern of proteins from 1.7 M NaCl-grown cells before delipidation is shown in lane a. The remaining lanes show fluorograms of protein gels prepared using cells harvested and delipidated after the following treatments: b, 1.7 M NaCl-grown cells labeled with [^{3}H]14:0; c, 3.0 M NaCl-grown cells labeled with [^{3}H]14:0; d, 1.7 M NaCl-grown cells labeled with [^{3}H]16:0; e, 3.0 M NaCl-grown cells labeled with [^{3}H]16:0. Equal amounts of individual samples (50 μg) were loaded onto the gels (see "Materials and Methods").

carried the heaviest labeling, but in 3.0 M NaCl this was not the case. Also, a pair of bands at about 17.2 and 15.8 kD were equally labeled in the 1.7 M NaCl cells but not in the 3.0 M NaCl cells (Fig. 1). The relative magnitude of these differences is more apparent in densitometry tracings (Fig. 2).

In contrast to the labeling pattern with [^{3}H]14:0, [^{3}H]16:0 labeled mainly low mol wt material in the 9 kD range (Fig. 1). Electrophoresis of the delipidated proteins on 20% acrylamide gels confirmed the about 9 kD mol wt and established that the labeled material was not simply free lipid.

Identification and Quantitation of Fatty Acids Linked to *Dunaliella* Acylated Proteins

To identify the chain lengths of fatty acids present in linkage with total protein, fatty acid methyl esters released from delipidated protein by acid methanolysis were analyzed by GLC. Table I shows the mean weight percentages of the fatty acids released. In samples obtained from either 1.7 or 3.0 M NaCl grown cells, the bulk of acylation is due to 14:0 (79% in proteins of 1.7 M cells and 93% in proteins of 3.0 M cells). The second most abundant fatty acid in each case is 16:0 (11 and 4%, respectively, for 1.7 and 3.0 M growth conditions). Traces of 18:0, 18:1, and 18:2 acids were also detected. In some experiments, a known amount of heptadecanoic acid (17:0) was included with the sample during acid methanolysis to serve as an internal standard for quantitative gas chromatography. This led to an estimate of approximately 10^{-2} μg fatty acid/μg protein in cells grown in both salt concentrations.

HPLC coupled with continuous scintillation counting of the eluate was used to determine the incorporation pattern of added [^{3}H]14:0 and [^{3}H]16:0 (as well as their elongation and

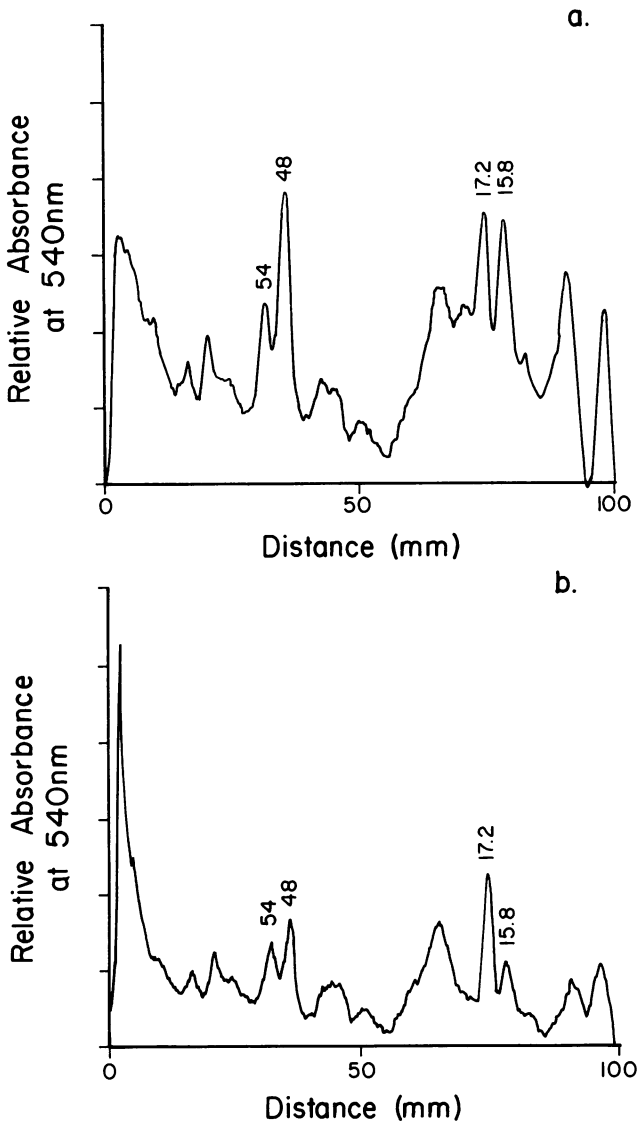

Figure 2. Densitometry tracings of fluorograms made from protein gels of: a, 1.7 M NaCl-grown cells labeled with [^{3}H]14:0 (gel b of Fig. 1) and b, 3.0 M NaCl-grown cells labeled with [^{3}H]14:0 (gel c of Fig. 1). Mol wt of selected proteins are shown for comparing the two tracings.

Table I. *GLC Identification of Fatty Acids Bound to Dunaliella Acylated Proteins*

Results are the mean of three independent experiments.

Fatty Acid	$\bar{X}$ Weight % ± SD	
	1.7 M	3.0 M
14:0	79 ± 5	93 ± 2
16:0	11 ± 4	4 ± 2
18:0	4 ± 2	2 ± 1
18:1	4 ± 1	1
18:2	1 ± 1	0

desaturation products) in *Dunaliella* acylated proteins under moderate and high-salt concentrations. The disposition of added [^{3}H]14:0 was nearly the same in acylated proteins of cells grown either in 1.7 or 3.0 M NaCl medium. In three experiments with 1.7 M NaCl-grown cells, an average of 56% of the label remained in 14:0 and 40% was found in 16:0. In 3.0 M NaCl-grown cells, 67% of the label was in 14:0 and 27% in 16:0. Small percentages of label (2–4%) appeared in 18:1 and 18:2, but no radioactivity was associated with 18.0. When [^{3}H]16:0 was administered to cells growing in medium containing 1.7 M NaCl, approximately 83% of the protein bound radioactivity was found in 16:0 and 7% in 18:0. No evidence of retroconversion of 16:0 to 14:0 was observed in any of the replicate experiments, in agreement with earlier findings (11). In the case of acylated proteins obtained from cells grown in medium containing 3.0 M NaCl, an average of 98% of the radioactivity released from the delipidated protein was found in 16:0 with 1.5% in 18:0. No label was detected in 14:0, 18:1, or 18:2. Unexpectedly, in each replicate set of experiments performed using exogenously added [^{3}H]16:0, 6% of the radioactivity was eluted with the retention time appropriate for 16:1. However, gas-liquid chromatographic analyses failed to detect a measurable mass of 16:1 in any of the samples examined, and further work will be needed to confirm the identity of this labeled component.

Labeled, delipidated protein treated with 1 N KOH released only 1% of its associated radioactivity, indicating a high percentage of alkali-stable bonds. This was not unanticipated, given the high content of 14:0 in *Dunaliella* acylated proteins and the findings of other workers that in many systems, myristate is almost exclusively found in alkali-stable amide linkage with protein (12). Some of the [^{3}H]14:0 labeled, delipidated proteins were treated with a phosphatidylinositide-specific phospholipase C in order to determine if a protein-linked glycophospholipid anchor (5) was present. Using the procedure of Ferguson *et al.* (3), which in our hands released >40% of protein-linked radioactivity from *Tetrahymena* preparations (PE Ryals, GA Thompson, Jr, unpublished observations), no significant amount of protein-associated radioactivity was released from *Dunaliella* preparations.

Our analyses of *Dunaliella* acylated proteins reveal a pattern of fatty acid attachment generally similar to that found in other organisms. At this time we cannot rigorously exclude the possibility that some of the covalently bound fatty acids are attached to nonprotein macromolecules, but coincidence of most of the major labeled regions on electrophoretic gels with Coomassie blue-staining bands, at least in the [^{3}H]14:0 labeled cells (Fig. 1), suggests that acylated proteins are the major labeled compounds present. The fatty acids in linkage with protein are mainly saturated, and the great majority of them appear to be in alkali-resistant amide linkage, with only about 1% esters present. This large proportion of amide bonds is similar to the situation in *Physarum polycephalum* (10), where there is an apparent absence of ester-bound acyl chains. A relatively small proportion (0.2%) of the incorporated radioactivity is found in linkage with *Dunaliella* protein. Others have reported protein-bound fatty acids ranging from 0.01% (15) up to 6.8% (8) of the total fatty acid content.

It is of particular interest to find that the acylation pattern of *Dunaliella* proteins is altered by the NaCl content of the growth medium. Because the cells grow only half as fast in the 3.0 M NaCl medium as in the lower salt concentrations, it is probable that they are under stress in the 3.0 M NaCl.

These differential rates of protein acylation may prove of value in gaining an understanding of *Dunaliella*'s osmoregulatory mechanisms and its ability to retain high internal concentrations (4.0 M) of glycerol (1). In a broader sense, the observed environmental effects on protein acylation in these cells provides another tool by which to examine the mechanisms governing protein acylation in general. The means by which nutrient composition, temperature and, in particular, light affect protein acylation in photosynthetic cells may now be systematically studied.

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